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Impact of mico-seed cookies with arbuscular mycorrhizal fungi on the growth, yield, and chemical composition of *Gynura procumbens*

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The mico-seed cookies incorporated arbuscular mycorrhizal fungi (AMF) and functioned as an organic conditioner in the soil to enhance nutrient availability through symbiotic impact on the plant at the root system level. The aim of the research was to evaluate the effect of mico-seed cookies on the growth rate, yield, and forage chemical characteristics of *Gynura procumbens.* The research was conducted in a randomized complete block design with four treatments of mico-seed cookies application (a control group (without mico-seed cookies), and three groups that used mico-seed cookies with diameters of 4 cm (53 g), 5 cm (84 g), and 6 cm (130 g)). The research site was located at IPB University in Bogor, Indonesia, on soil with an acidic pH range of 3.93-4.45. The results showed that the mico-seed cookie improved the direct planting system of *G. procumbens* indicated by the increasing seedling rate, growth rate, and forage yield. Additionally, the mico-seed cookies triggered the bioactive compounds production. The research concluded that the application of the mico-seed cookie direct planting system to *G. procumbens* resulted in a statistically significant enhancement of the seedling percentage (up to 15%), colonization (up to 70%), growth rate (25-30%), yield (42-44%), and forage chemical characteristics, especially on trigger bioactive compounds. *G. procumbens* has potential as forage based on biomass production and high-quality nutrient, especially leaf protein content that reaches up to 25%.
Keywords: Arbuscular mycorrhizal fungi, biomass production, forage quality, growth rate, *Gynura procumbens,* mico-seed cookies.

INTRODUCTION

Trees and shrubs have been utilized as an essential forage for local farmers in Indonesia (Kumalasari *et al.,* 2022). One of the indigenous shrubs, *Gynura procumbens* (Lour.) Merr., which is endemic to Southeast Asia and widely distributed throughout Malaysia, Indonesia, Thailand, and China (Bari *et al.,* 2021), has been studied as an ethnomedicinal in several countries (Hew *et al.* 2010). However, the adoption of this plant as a forage has been hindered by limited forage production and nutrient content. The nutrient content of *G. procumbens* is crucial information in optimizing forage utilization on animals, given the significant impact of nutrient levels on animal performance (Maña *et al.,* 2023).

Forage production in Indonesia is primarily limited to areas with marginal conditions such as acidic soil, saline areas, or former mining sites (Syafitri and Nasrullah, 2020). The introduction of *G. procumbens* as forage have to face challenges of poor soil conditions and the minimum information of both biomass production and nutrient content. Furthermore, the irregular supply of regenerative materials for tissue culture propagation in Indonesia complicates the utilization of this method for reproduction (Khaniyah *et al.,* 2012). Consequently, forage crops necessitate a nursery to meet the physical standards, including land area, water sources/irrigation systems, utilities, and supporting facilities, as well as human resources to ensure successful propagation. Plant nurseries play a vital role in the germination, growth, and seedling adaptation of plants until they are strong and

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healthy enough to be transplanted or relocated to planting sites (Garba *et al.*, 2019). The establishment of forage plant nurseries is more costly than ornamental plant nurseries, as they require bulk plants, which are more expensive.

An innovative plant media, in the form of mico-seed cookies, has been designed to provide plant nutrients and reduce the cost of forage plant nurseries. These cookies contain arbuscular mycorrhizal fungi (AMF) and function as an organic conditioner in the soil, increasing nutrient availability through symbiotic impact on the plant at the root system level (Prihantoro *et al.*, 2023). The effectiveness of AMF conditioners has been proven on *Indigofera zollingeriana* nurseries (Pratama *et al.*, 2023). Other researchers have reported that AMF conditioners increased the growth of *Clitoria ternatea* on saline soil (Rizki *et al.*, 2023), *Sorgum bicolor* (Fitria *et al.*, 2022) and *Stylosanthes guianensis* on acid soil (Rizki *et al.*, 2022) and dry land (Marzukah *et al.*, 2023).

The investigation of *G. procumbens* with regards to direct planting in acidic soil is imperative for enhancing the forage supply for animal livestock purposes. Hence, the primary objective of this research was to evaluate the effect of mico-seed cookies on the growth rate, yield, and forage chemical characteristics of *G. procumbens*.

MATERIALS AND METHODS

***Location*:** The research was conducted in the experimental field of the Division of Forage Science and Pasture Technology, Department of Nutrition and Feed Technology, Faculty of Animal Science, at IPB University (2022-2023). The experimental field was located at 6°33'10.624" S latitude and 106°43'21.719" E longitude at an altitude of 202 meters above sea level. The soil used in the study is presented in Table 1 and has the following agrochemical characteristics acidic soil (pH 3.93-4.45) with low mineral content and tends to clay type.

***Materials*:** The research was conducted on an area of 750 m^2 using a randomized complete block design (RCBD) with 10 replications. The study involved two factors: 4 levels of mico-seed cookies for direct planting and 3 sequential cuts. The treatments consisted of a control group (without mico-seed cookies), and three groups that used mico-seed cookies with diameters of 4 cm (53 g), 5 cm (84 g), and 6 cm (130 g) (modified from Alfain *et al.* 2023). Each block consisted of 22 rows, with a length of 13 meters, and a spacing of 50 cm between rows and 150 cm between blocks. For this research, stem cuttings were collected from the mature plant of *G. procumbens* (2 years old) with a stem age was 6 months after pruning. A total of 176 cutting stems of *G. procumbens*, with a length of 30 cm, were cultivated in the experimental site after plowing at 20 cm and hoeing at 10 cm.

***Methods*:** The mico-seed cookies were prepared in the form of flat rounds like cookies, with sizes corresponding to the treatments. All of the *G. procumbens* cutting stems were planted by inserting the mico-seed cookies above the soil and then pushing them down until they were 10 cm below the soil surface. The days from the date of cultivation to the emergence of buds on the planted cutting stems were recorded as the days to emergence. The seedlings percentage was determined by counting the number of cutting stems that had grown per total cutting stems in the plot, 30 days after cultivation. Ten plants were randomly selected in each experimental plot, and their heights were measured from the ground to the tip of the plant at the time of cutting. The number of branches and leaves was then counted. To determine the fresh biomass, the accessions of vetch were harvested at a height of 30 cm in the 12 weeks after the first cultivating

Table 1. The soil characteristics of the experimental field.

Parameters	Methods	Result	Unit
pH	H_2O	4.45	
	KCl	3.93	
C-org	Walkley and Black	1.47	%
N-total	Kjeldahl	0.13	%
P	Bray I	0.95	ppm
	HCl 25%	84.70	ppm
Ca	*N* NH_4OAc pH 7.0	1.41	cmol$^{(+)}$/kg
Mg	*N* NH_4OAc pH 7.0	0.27	cmol$^{(+)}$/kg
K	*N* NH_4OAc pH 7.0	0.09	cmol$^{(+)}$/kg
Na	*N* NH_4OAc pH 7.0	0.10	cmol$^{(+)}$/kg
KTK	*N* NH_4OAc pH 7.0	13.70	cmol$^{(+)}$/kg
Base saturation		13.58	%
Al	*N* KCl	2.40	
H	*N* KCl	0.70	cmol$^{(+)}$/kg
Fe	*DTPA*	42.40	ppm
Cu	*DTPA*	1.57	ppm
Zn	*DTPA*	2.69	ppm
Mn	*DTPA*	1.41	ppm
Soil texture	Pipette method		
Sand		7.85	%
Silt		15.49	%
Clay		76.66	%

. The roots were extracted for the purpose of identifying the type of mycorrhizal infection present, specifically whether it was characterized by vesicles, spores, or hyphae. The second and third cuttings were conducted at a height of 5 cm above the first cutting in 8 weeks. The weight of the total fresh biomass yield was recorded from each plant in the field, and a sample was oven-dried for 72 hours at a temperature of 70^{o}C. The oven-dried samples were weighed to determine the total dry matter yield. The dry matter and crude fibre were analyzed using SNI 01.2891-1992, while the ash, crude protein, and extract ether content were analyzed according to AOAC method (2005). The data were analyzed using R i386 3.6.1 with packages Rcmdr, agricolae, car, and emmeans. The

analysis of variance (ANOVA) was utilized to examine the impact of mico-seed cookies on plant establishment, agronomical traits, and forage yield, with the data separated by Tukey Contrast. Conversely, descriptive methods were employed to analyze the nutrient content, bioactive compounds, and amino acid content of the *G. procumbens* forage.

RESULTS

***The effect of mico-seed cookies on plant establishment*:** The research revealed that mico-seed cookies have an impact on the early germination phase of *G. procumbens*. The application of thicker mico-seed cookies resulted in a decrease in the number of days to emergence and an increase in the seedling percentage (as shown in Table 2). Additionally, there were statistically significant differences in the number of days to plant emergence with increasing mico-seed cookie diameter. The results indicated that wider mico-seed cookies decreased the number of days to emergence from 7.39 days to 6.73 days and increased the seedling percentage to 100%.

***The effect of mico-seed cookies on plant agronomical traits in three cutting times*:** The results indicated that the pattern of plant height, number of branches, and leaves varied in response to the addition of mico-seed cookies and cutting time. The application of mico-seed cookies led to an increase in plant height and number of leaves (Table 2).

Table 2. The effect of mico-seed cookies on cutting stem emergence and seedling percentage.

Treatment	Days to emergence	Seedling percentage (%)	Colonization (%)
M0	7.39b	85.17c	6.10c
M1	7.23b	92.59b	44.11b
M2	6.85ab	98.14ab	69.85ab
M3	6.73a	100.00a	77.17a
Mean	7.05	93.98	49.31
SEM	0.48	2.21	13.04

Data are represented as means; SEM: standard error of means (n=176); M0: control; M1: mico-seed cookies with diameters of 4 cm (53 g); M2: mico-seed cookies with diameters of 5 cm (84 g); and M3: mico-seed cookies with diameters of 6 cm (130 g); Mean with different letters in the same column are statistically different ($p<0.05$) according to Tukey test.

Furthermore, it was observed that the height of *G. procumbens* plants was significantly increased when cultivated with higher doses of mico-seed cookies. Specifically, the tallest plant (77.57 cm) was observed in the treatment that received 130 g of mico-seed cookies, while the rest of the treatments had lower heights. On the first and second cutting times, the mean plant height was higher than on the third cutting time, which reached 54.42 cm. Additionally, the number of leaves increased with the size of the mico-seed cookie application on the cutting stem and over the cutting time.

The branch data displayed contrasting trends in mico-seed cookie application and cutting time. There were no discernible changes in the number of branches across different mico-seed cookie applications, but for the third cutting time, the number of branches more than tripled in comparison to the previous measurement. Initially, the number of branches was generally low, but during the third subsequent growth period, the rate of branch accumulation accelerated significantly. As a result, the multiplication of branches led to an augmentation in the number of leaves, which reached a total of 126.42 leaves.

Table 3. The effect of mico-seed cookies on plant height, number of branches and leaves of *G. procumbens* in three cutting times.

Treatments	Plant height	Number of branches	Number of leaves
Mico-seed cookies			
M0	57.73b	9.83	80.53b
M1	58.31b	9.78	89.72ab
M2	63.54b	10.23	91.58ab
M3	77.57a	10.21	107.40a
SEM	2.24	0.85	5.92
Harvest time			
Y1	66.27b	3.70c	47.62b
Y2	78.02a	5.38b	36.49b
Y3	54.42c	17.67a	126.42a
SEM	1.24	0.43	3.01
Interaction of mico-seed cookies × harvest time			
M0Y1	57.89c	3.89	41.35bc
M0Y2	63.56bc	3.68	36.40c
M0Y3	53.79c	18.05	109.39a
M1Y1	60.32bc	3.90	43.70c
M1Y2	62.95bc	5.25	37.83c
M1Y3	53.91c	17.28	124.25a
M2Y1	69.06b	3.56	47.93bc
M2Y2	72.54b	5.54	36.20c
M2Y3	53.76c	18.05	126.05a
M3Y1	77.24b	3.46	57.10b
M3Y2	113.62a	7.12	35.54c
M3Y3	56.18c	17.30	145a
SEM	3.48	1.28	8.92

Data are represented as means; SEM: standard error of means (n=176); M0: control; M1: mico-seed cookies with diameters of 4 cm (53 g); M2: mico-seed cookies with diameters of 5 cm (84 g); and M3: mico-seed cookies with diameters of 6 cm (130 g); Y1: the first cutting time (12 weeks); Y2: the second cutting time (8 weeks); Y3: the third cutting time (8 weeks)Mean with different letters in the same column are statistically different ($p<0.05$) according to Tukey test.

***The effect of mico-seed cookies on forage yield*:** Table 4 displays notable discrepancies in forage yield based on cutting time and the mico-seed cookies treatments. The mico-seed cookies application resulted in a substantial increase in biomass production, nearly double that of the control group. The *G. procumbens* biomass production dynamics were observed to occur on cutting time, with the highest production recorded on the third cutting occasion. This was due to the branches' growth, which resulted in the accumulation of more leaves compared to the first and second cutting times (Table 3).

Table 4. The effect of mico-seed cookies on fresh forage yield (ton/ha).

Treatments	Fresh biomass	Dry biomass
Mico-seed cookies		
M0	104.11c	57.26c
M1	114.18c	63.94bc
M2	139.89b	75.54b
M3	182.21a	103.86a
SEM	13.44	7.46
Harvest time		
Y1	104.79b	59.210b
Y2	72.39b	41.991b
Y3	195.68a	111.538a
SEM	7.01	4.010
Interaction of mico-seed cookies × harvest time		
M0H1	69.95c	38.477c
M0H2	67.94c	38.046c
M0H3	150.63b	81.344b
M1H1	88.35bc	50.360c
M1H2	73.13c	40.322c
M1H3	158.09ab	89.325b
M2H1	120.49b	69.885bc
M2H2	72.32c	41.226c
M2H3	195.00a	111.150ab
M3H1	138.01b	75.908b
M3H2	76.24c	43.078c
M3H3	278.98a	161.812a
SEM	20.45	11.661

Data are represented as means; SEM: standard error of means (n=176); M0: control; M1: mico-seed cookies with diameters of 4 cm (53 g); M2: mico-seed cookies with diameters of 5 cm (84 g); and M3: mico-seed cookies with diameters of 6 cm (130 g); Y1: the first cutting time (12 weeks); Y2: the second cutting time (8 weeks); Y3: the third cutting time (8 weeks)Mean with different letters in the same column are statistically different ($p<0.05$) according to Tukey test.

The research results indicated that the interaction of addition of mico-seed cookies and plants age significantly influenced the biomass of the older plants, leading to an average biomass higher than that of the younger plants without mico-seed cookies. Specifically, the highest amount of biomass was observed in the third harvest with mico-seed cookies placed at a distance of 6 cm. Additionally, the study revealed that as the plants aged, the distance between them expanded.

***The effect of mico-seed cookies on nutrient content*:** Table 5 displays the average nutrient content of *G. procumbens*, which was harvested at 8-12 weeks after planting. The crude protein content of each treatment was not significantly varied, yet all contained a high level of crude protein (greater than 25%). This high protein content in *G. procumbens* leaves signifies the plant's potential as a protein source for ruminants. Additionally, the low crude fiber content and high ash content suggest a high mineral content in the plant material.

Table 5. Nutrient content (%).

Treat.	DM	Ash	Crude protein	Crude fibre	Ether extract	Beta-N
M0	28.95	10.87	25.68	9.91	2.13	56.56
M1	27.78	9.49	25.34	14.95	2.18	51.15
M2	30.39	6.55	25.57	15.88	2.22	55.20
M3	28.92	9.60	26.17	16.31	1.88	50.49

M0: control; M1: mico-seed cookies with diameters of 4 cm (53 g); M2: mico-seed cookies with diameters of 5 cm (84 g); and M3: mico-seed cookies with diameters of 6 cm (130 g).

***The effect of mico-seed cookies on bioactive compounds*:** The significance of bioactive compounds in forage production cannot be overstated, as their influence on animal nutrition is of utmost importance (Tava *et al.*, 2022). The research result has demonstrated that mico-seed cookies have a significant impact on the formation of these compounds in *G. procumbens*, with the presence of quinone and triterpenoids being noted in the cookies, and their absence in the control or smaller size cookies. The quantitative analysis found that contained high levels of phenol (0.53-0.70) and flavonoids (1.75-2.34).

Table 6. Bioactive compounds in *G. procumbens.*

Treat.	Alkaloid	Tanin	Saponin	Quinone	Steroid	Triterpenoid	Fenol	Flavonoid
M0	-	+	+	-	+	-	0.53	2.32
M1	-	+	+	-	+	-	0.70	1.75
M2	-	+	+	+	+	+	0.55	1.89
M3	-	+	+	+	+	+	0.62	2.34

M0: control; M1: mico-seed cookies with diameters of 4 cm (53 g); M2: mico-seed cookies with diameters of 5 cm (84 g); and M3: mico-seed cookies with diameters of 6 cm (130 g).

Amino acids content in *G. procumbens*: Table 7 displays the amino acid profile of *G. procumbens*. The analysis revealed that the concentration of amino acids in *G. procumbens* exhibited considerable variation across all amino acids.

Table 7. Amino acid content in *G. procumbens* (g/100g).

Amino acid	Average	Amino acid	Average
Aspartic acid	1.41±0.015	Tyrosine	0.42±0.005
Glutamic acid	2.16±0.025	Valine	0.79±0.000
Serine	0.40±0.010	Methionine	0.38±0.005
Glycine	0.68±0.035	Cysteine	0.35±0.055
Histidine	0.43±0.005	Isoleucine	0.58±0.035
Arginine	0.74±0.005	Leucine	0.96±0.020
Threonine	0.34±0.045	Phenylalanine	0.84±0.035
Alanine	0.80±0.020	Lysine	0.68±0.030
Proline	0.53±0.015		

DISCUSSION

The poor soil condition forces the *G. procumbens* plant to associate with microorganisms in the interior tissues of plant parts to increase the photosynthetic rate that was presented by 6.10% colonization on control treatment. The cookies diameter size was enough to facilitate the wider diameter cutting stem size of *G. procumbens* (Alfain *et al.,* 2023). Thus, the colonization was formed well in the roots that grew in marginal soil, which is a part of the plant's defense mechanism in unfavorable environments (Sianturi *et al.,* 2015). As a result, the application of mico-seed cookies increased the colonization percentage on *G. procumbens* roots. The use of mico-seed cookies on *G. procumbens* promoted the infection of arbuscular mycorrhizae (44.11 to 77.17%), as confirmed by microscopy, which revealed vesicula, spores, and hyphae.

The arbuscular mycorrhizae infection could enhance the efficiency of *G. procumbens* root function in absorbing soil nutrients, thereby promoting plant growth (Marzukah *et al.,* 2023). This may be due to the increasing leaf chlorophyll and proline levels that support biomass production in acid soil (Rizki *et al.,* 2022). However, the impact of micro-seed cookies on branch development is unrelated to trends in diameter growth, as the development of plant branches is primarily controlled by genetic and hormonal factors (Yang *et al.,* 2022). According to Mutetwa and Mtaita (2014), organic fertilizers have a slower effect than inorganic fertilizers because the organic fertilizers release required nutrients at a slower rate. The arbuscular mycorrhizae serve as a soil conditioner, balancing soil microorganisms to optimize nutrient absorption (Pratama *et al.,* 2023). These conditions were confirmed in the third planting cycle, where the number of branches was tripled compared to the previous cycle (Table 4). This result supports the findings of Gutowski (2015), who reported that applying arbuscular mycorrhizae to radish seeds could increase seed germination due to improved nutrient uptake.

The nutrient content of *G. procumbens* was found to be unaffected by the application of mico-seed cookies on mico-seed cookies application due to an imbalance of minerals in the soil (Diagne *et al.,* 2020) or low soil fertility that application of both organic and inorganic fertilizers had no direct effect on forage crude protein (Kakabouki *et al.,* 2014). However, the crude protein content in the present research was higher than in other plants such as napier grass (Budiman *et al.,* 2012), cowpea, mungbean, and guar fodder (Ghotbi *et al.,* 2022).

Moreover, El-Amier *et al.* (2022) found that the application of mico-seed cookies may also increase the suitability of the soil for the formation of bioactive compounds due to the availability of nutrients in the soil and its properties that support forage growth and bioactive compound production. Mico-seed cookies play a role as a soil conditioner and provides the soil microclimate to supply enough growth hormones that affect the bioactive compounds of *G. procumbens* (Pramita *et al.,* 2018). The production of bioactive compounds has the potential to enhance nutrient utilization efficiency in ruminants, reduce the risks of mycotoxin parasitism, and improve animal health and performance (Niderkörn and Jayanegara, 2020). Therefore, the use of mico-seed cookies in forage production is a promising approach to increasing the production of bioactive compounds and improving animal nutrition.

The determination of the optimizing level of animal performance is contingent upon the association between amino acids in forage and dietary energy (Titgemeyer and Löest, 2001). Recent research has indicated that the concentration of amino acids in *G. procumbens* is lower than in other forages, including perennial ryegrass, white clover, a ryegrass/white clover sown sward, and alfalfa (Edmund *et al.,* 2013). However, it is higher than in other forages, especially grasses (Li *et al.,* 2012). The variation in amino acid content can be attributed to both the species and mixture of forage (Ates, 2012) and the plant environment conditions (Ahmed *et al.,* 2021).

The research revealed that mico-seed cookies have the potential to enhance the seedling percentage, microorganism colonization on soil, leading to an increase in the number of leaves, plant biomass, and the production of bioactive compounds. Furthermore, *G. procumbens* was found to produce high biomass with a high nutrient content, rich bioactive compounds, and amino acids. The application of mico-seed cookies on *G. procumbens* was observed to accelerate the number of branches and biomass on the third plant growth. The utilization of mico-seed cookies as a soil conditioner may require a significant amount of manual labor to produce and apply them to extensive areas. Similarly, the incorporation of *G. procumbens* as forage may be limited due to the palatability of the forage and the time required for animals to adapt to it.

Conclusion**:** The application of the mico-seed cookie direct planting system to *Gynura procumbens* resulted in a statistically significant enhancement of the seedling percentage (up to 15%), colonization (up to 70%), growth rate

(25-30%), yield (42-44%), and forage chemical characteristics, especially on trigger bioactive compounds. *G. procumbens* has potential as forage based on biomass production and high-quality nutrient, especially leaf protein content that reaches up to 25%.

***Authors contributions statement*:** N.R. Kumalasari, P.D.M.H. Karti designed the experiments, W.S. Mulyati completed the experiments; N.R. Kumalasari, Sunardi prepared the draft; D.M. Fassah, R.S.H. Martin, P.S. Negoro reviewed and finalized the draft.

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